

## A Novel mRNA–cDNA Interference Phenomenon for Silencing bcl-2 Expression in Human LNCaP Cells

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The templates required for inducing posttranscriptional gene silencing (PTGS) effects have been investigated in human prostate cancer LNCaP cells. Transfection of a mRNA–cDNA hybrid construct was found to result in a relatively long-term interference of specific gene expression. Androgen-stimulated expression of bcl-2 has been reported to increase the tumorigenic and metastatic potentials of human prostate cancer LNCaP cells, as well as their resistance to many apoptotic stimuli. The addition of bcl-2 antisense oligonucleotides, however, restored apoptosis. Our studies demonstrate gene silencing effects of the mRNA–cDNA transfection that is similar to those of PTGS/RNAi in this *in vitro* prostate cancer cell model. A potential RNA-directed RNA polymerase activity was also detected which is  $\alpha$ -amanitin-sensitive. These findings indicate that a novel gene silencing system may exist in mammalian cells. © 2001 Academic Press

**Key Words:** mRNA–cDNA interference phenomenon (D-RNAi); posttranscriptional gene silencing (PTGS); RNA-directed RNA polymerase (RaRp); prostate cancer cells; bcl-2.

Ever since 1998, posttranscriptional gene silencing (PTGS) and RNA interference (RNAi) have been found to be capable of quelling special gene activities in a variety of *in-vivo* systems, including plants (1), *Drosophila melanogaster* (2–4), *Caenorhabditis elegans* (5–8), zebrafish (9), and mouse (10). In general, the transfection of a plasmid-like DNA structure (transgene) into cells induces PTGS phenomena, while that of a double-stranded RNA (ds-RNA) causes an RNA interference (RNAi) effect. The introduction of a trans-

gene or ds-RNA probably evokes a process of an intracellular sequence-specific RNA degradation affecting all highly homologous transcripts, which is referred to as cosuppression.

It has been proposed that cosuppression results from the generation of small RNA products (21–25 nucleotide bases) by an RNA-directed RNA polymerase (RdRp) (1) and/or a ribonuclease (RNase) activity (6, 11, 12). This type of generation of small RNA products is similar to that induced by aberrant RNA templates frequently generated from the transfecting nucleic acids or viral infection. However, it is not clear whether the small RNAs are directly produced by RdRp (1) or whether they are indirectly cleaved from larger RdRp-derived RNA precursors by a specific RNase (6, 11). Apart from the fact that an RdRp-independent endoribonucleolysis model has been reported for the effect of RNAi in *Drosophila* (12), RdRp homologues are widely found in *Arabidopsis thaliana* as Sde-1/Sgs-2 (13), *Neurospora crassa* as Qde-1 (14) and *Caenorhabditis elegans* as Ego-1 (15). These RdRp homologues are capable of and required for maintaining a long-term/inheritable PTGS/RNAi effect (11).

Although transfection of transgene/ds-RNA has been shown to produce PTGS/RNAi effects in plants and in some simple animals, there is no strong evidence for this occurring in mammalian cells. The objective of this study was to determine whether a potentially RdRp-dependent novel gene silencing phenomenon, similar to the PTGS/RNAi observed in other species, exists in human prostate cancer cells. To examine alternative PTGS/RNAi induction in human cancer cells, we have tested several different RNA/DNA oligonucleotide constructs for their ability to prevent the expression of specific oncogenic genes. For operational purposes, this approach is termed DNA–RNA interference (D-RNAi).

We took advantage of the fact that normal human prostatic secretory epithelial cells do not express bcl-2 proteins, whereas a number of neoplastic prostate tis-

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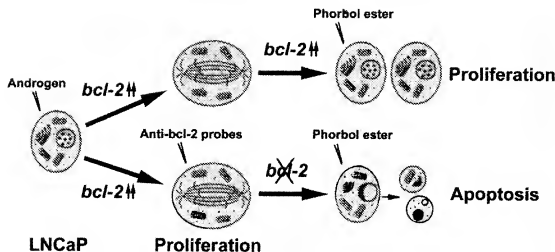


FIG. 1. Schematic illustration of experimental procedures for testing interference of bcl-2 gene expression in androgen-treated human prostate cancer LNCaP cells.

issues from androgen-ablation patients showed an elevated level of these apoptosis-suppressing oncoproteins (16). Moreover, over-expression of bcl-2 was known to protect prostate cancer cells from apoptosis *in vitro* and confer resistance to androgen depletion *in vivo* (16, 17). The tumorigenic and metastatic potentials of LNCaP cells were also significantly increased after bcl-2 stimulation by either androgen or transgene treatment (16, 18, 19). Such inhibition of apoptosis can be prevented by the treatment of bcl-2 antisense oligonucleotides but not those of other apoptotic stimuli such as etoposide or phorbol ester (16, 18). Accordingly, LNCaP cells were treated with dehydrotestosterone (20 nM 5 $\alpha$ -arostan-17 $\beta$ -ol-3-one) to block apoptosis induced by phorbol ester (10 nM phorbol-12-myristate-13-acetate) and then, an anti-bcl-2 D-RNAi was introduced in order to restore the apoptosis in these prostate cancer cells (Fig. 1).

Our study demonstrates that ectopic transfection of a sequence-specific messenger RNA (mRNA)-complementary DNA (cDNA) hybrid (D-RNAi), rather than an antisense RNA (aRNA)-cDNA or a ds-RNA construct, induces specific intracellular gene silencing in human cells. We have successfully detected specific gene interference of bcl-2 expression by D-RNAi in human LNCaP prostate cancer cells. The application of D-RNAi might therefore have significant therapeutic potential in other varieties of cancer cells of preventing a specific gene expression, such as bcl-2, increasing the susceptibility of these cancer cells to apoptotic stimuli, and thus reducing tumor growth.

## MATERIALS AND METHODS

**Oligonucleotides.** Four synthetic oligonucleotides were used in the generation of bcl-2 RNA-DNA hybrids as follows: T7-bcl2 primer (5'-dAAACGACGCCAGTGAATTGTAATACGACTCACTATAG-

CGGGATGACTGAGTACCTGAACCGGC-3') and anti-bcl2 primer (5'-dCTTCTTCAGGCCAGGGAGCCATGG-3') for mRNA-cDNA hybrid (D-RNAi) probe preparation; T7-anti-bcl2 primer (5'-dAAACGACGCCAGTGAATTGTAATACGACTCACTATAGGCC-TTCTTCAGGCCAGGGAGCCATGG-3') and bcl2 primer (5'-dGGATGACTGAGTACCTGAACCGGC-3') for antisense RNA (aRNA)-cDNA hybrid (reverse D-RNAi) probe preparation. The design of the sequence-specific primers is based on the same principle used by PCR (50–60% G-C rich), while that of the promoter-linked primers, however, requires a higher G-C content (60–65%) working at the same annealing temperature as above sequence-specific primers due to their unmatched promoter regions. For example, new annealing temperature for the sequence-matched region of a promoter-linked primer is equal to  $[2^{\circ}\text{C} \times (\text{dA} + \text{dT}) + 3^{\circ}\text{C} \times (\text{dC} + \text{dG})] \times 5/6$ , not including the promoter region. All primers need to be purified by polyacrylamide gel electrophoresis (PAGE) before used in a RNA-PCR reaction.

**General methods.** All routine techniques and DNA manipulations, such as gel electrophoresis, were performed according to standard procedures (20). All enzymes and buffer treatments were applied following the manufacturer's recommendations (Roche Biochemicals, Indianapolis, IN). For Northern blots, mRNAs were fractionated on 1% formaldehyde-agarose gels and transferred onto nylon membranes (Schleicher & Schuell, Keene, NH). Probes were labeled with the Prime-It II kit (Stratagene, La Jolla, CA) by random primer extension in the presence of  $[^3\text{P}]\text{dATP}$  (>3000 Ci/mM, Amersham International, Arlington Heights, IL), and purified with Micro Bio-Spin chromatography columns (Bio-Rad, Hercules, CA). Hybridization was carried out in the mixture of 50% freshly deionized formamide (pH 7.0), 5 $\times$  Denhardt's solution, 0.5% SDS, 4 $\times$  SSPE and 250  $\mu\text{g}/\text{ml}$  denatured salmon sperm DNAs (18 h, 42 $^{\circ}\text{C}$ ). Membranes were sequentially washed twice in 2 $\times$  SSC, 0.1% SDS (15 min, 25 $^{\circ}\text{C}$ ), and once each in 0.2 $\times$  SSC, 0.1% SDS (15 min, 25 $^{\circ}\text{C}$ ); and 0.2 $\times$  SSC, 0.1% SDS (30 min, 65 $^{\circ}\text{C}$ ) before autoradiography.

**Cell culture and treatments.** LNCaP cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD) and grown in RPMI 1640 medium supplemented with 10% fetal bovine serum with 100  $\mu\text{g}/\text{ml}$  gentamycin at 37 $^{\circ}\text{C}$  under 10%  $\text{CO}_2$ . Androgen withdrawal and resupplement were carried out as previously described (18) with minor modification. These cultured cells were treated with one dose of 20 nM 5 $\alpha$ -arostan-17 $\beta$ -ol-3-one to induce bcl-2 expression. For liposomal transfection of anti-bcl-2 probes, the probes (5 nM) in DOTAP liposome (Roche Biochemicals) were applied to a 60

mm culture dish which contained LNCaP cells at 15% confluency. After an 18-h incubation, the cells took up about 60% of the probe-containing liposome. Uptake improved to 100% after 36 h of incubation. The addition of  $\alpha$ -amanitin was completed at the same time as the liposomal transfection. The apoptotic effect of phorbol-12-myristate-13-acetate (10 mM) was initiated at 12 h after liposomal transfection. The mRNAs from the transfected LNCaP cells were isolated by poly-(dT) dextran columns (Qiagen, Santa Clarita, CA), fractionated on a 1% formaldehyde-agarose gel after a 36-h incubation period, and transferred onto nylon membranes. After 48-h transfection, genomic DNAs were isolated by an apoptotic DNA ladder kit (Roche Biochemicals) and assessed on a 2% agarose gel. The cell growth and morphology were also examined by both microscopy and cell counting as previously reported (21).

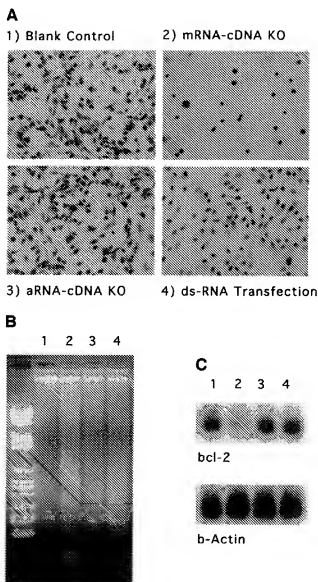
**Probe preparations.** For the generation of RNA-DNA hybrid probes, an RNA-polymerase cycling reaction (RNA-PCR) procedure was modified to generate either mRNA-cDNA or cDNA-aRNA hybrids (22). Total RNAs (0.2  $\mu$ g) from androgen-treated LNCaP cells were applied to a reaction (50  $\mu$ l in total) on ice, comprising 5  $\mu$ l of 10 $\times$  RT&T buffer (400 mM Tris-HCl, pH 8.3 at 25°C, 400 mM NaCl, 80 mM MgCl<sub>2</sub>, 2 M betaine, 100 mM DTT, and 20 mM spermidine), 1  $\mu$ M sequence-specific primer for reverse transcription, 1  $\mu$ M promoter-linked primer for cDNA-doublestranding, 2 mM rNTPs, 2 mM dNTPs and RNase inhibitors (10 U). After C. therm./Taq DNA polymerase mixture (4 U each) was added, the reaction was incubated at 52°C for 3 min, 65°C for 30 min, 94°C for 3 min, 52°C for 3 min, and then 68°C for 3 min. This formed a promoter-linked double-stranded cDNA for next step of transcriptional amplification up to 2000 fold/cycle. An *in vitro* transcription reaction was performed by adding T7 RNA polymerase (160 U) and C. therm. polymerase (6 U) into above reaction. After 1 h incubation at 37°C, the resulting mRNA transcripts were continuously reverse-transcribed into mRNA-cDNA hybrids at 52°C for 3 min and then 65°C for 30 min. The generation of cDNA-aRNA hybrids was the same procedure as aforementioned except using 1  $\mu$ M sequence-specific primer for cDNA-doublestranding and 1  $\mu$ M promoter-linked primer for reverse transcription. The RNA-PCR procedure can be reiterated to produce enough RNA-DNA hybrids for gene silencing analysis. For the preparation of double-stranded RNA probes, complementary RNA products were transcribed from both orientations of above promoter-linked double-stranded cDNAs and mixed together without reiterating reverse transcription activity. The quality of amplified probes were assessed on a 1% formaldehyde-agarose gel.

## RESULTS AND DISCUSSION

In order to examine alternative pathways for PTGS/RNAi induction in cancer cells, we have tested several oligonucleotide constructs for investigating their ability to prevent the expression of oncogenic genes. One of the approaches is based on a thermocycling RNA-PCR procedure to amplify highly concentrated RNA-DNA hybrids of a specific gene as reported previously (22). We have also examined the PTGS/RNAi requirement based on different orientations of the RNA and DNA constructs. Consequently, we have developed a type of template which is effective in producing gene silencing in human prostate cancer LNCaP cells.

### Effects of D-RNAi on Specific Gene Silencing in Human Prostate Cancer LNCaP Cells

To the best of our knowledge, this observation is the first time that D-RNAi was detected in human prostate



**FIG. 2.** Analysis of different templates for bcl-2 gene interference as follows: (1) blank control, (2) mRNA-cDNA hybrid, (3) aRNA-cDNA hybrid, and (4) ds-RNA in LNCaP cells. (A) The changes of cell proliferation rate and morphology. Chromosomal DNAs were stained by hematoxylin. Although the ds-RNA transfection also showed minor morphological changes, a significant inhibition of cell growth and chromatin condensation only occurred in the mRNA-cDNA transfection ( $n = 4$ ). (B) Genomic ladder patterns demonstrated the apoptosis induction of the bcl-2 mRNA-cDNA transfection. (C) Northern blots showed a strong gene silencing effect of the mRNA-cDNA transfection in bcl-2 expression.

cancer LNCaP cells, suggesting an effect of PTGS/RNAi in mammalian cells. Neither other workers nor our RNAi experiments showed a significant effect of gene silencing in mammalian cells when ds-RNA was used for transfections. However, when RNA-DNA hybrids larger than 500 bases were used, significant long-term (>6 days) effects of PTGS/RNAi-like gene silencing was detected in the mRNA-cDNA transfected cells 36-h after being treated with a single transfection.

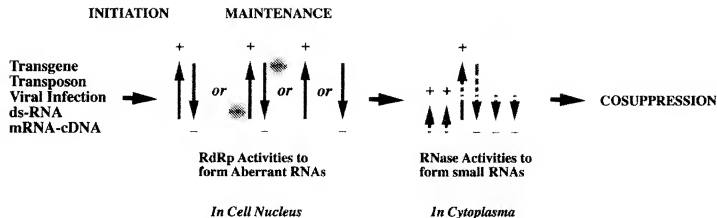


FIG. 3. Proposed model for long-term PTGS/RNAi/D-RNAi mechanisms. Initiation and maintenance periods varied depending on different biological systems and transfected genes. Although these mechanisms provide similar gene silencing results, the detailed molecular processes may be very distinct from each other.

while no effects or much less effect were observed in other parallel approaches.

As shown in Fig. 2, the transfection of bcl-2 mRNA-cDNA hybrids (5 nM) into LNCaP cells was sufficient to silence bcl-2 expression and cause apoptosis as determined by chromatin condensation and genomic DNA laddering fragmentation. However, no silence of bcl-2 expression was observed when double-stranded DNA, ds-RNA, or aRNA-cDNA hybrid was transfected. These findings indicate that a mRNA template and/or a RdRp-like enzyme is required for triggering the onset of a D-RNAi mechanism. Previously, the treatment of dehydrothiostosterone was shown to inhibit the apoptotic stimuli of phorbol ester and the addition of at least 40 mM antisense DNA probes abolished the inhibition of apoptosis (18, 23). Each transfection of the antisense DNA probes provided an effect of either fast (within 24-h incubation) or relatively short-term (2 to 3 days) gene knockout, therefore, the relatively long-term initiation and maintenance of D-RNAi could not be explained by antisense DNA probes themselves. Moreover, the concentration of mRNA-cDNA hybrids required for significant gene-silencing effects was about a half million fold less than those of antisense DNAs. Thus, the effectiveness of D-RNAi as reported here is not necessarily a result of the cDNA part of the mRNA-cDNA hybrid. For these reasons, we postulate that a RdRp-like enzyme may be involved in generating the precursors of small RNAs on the basis of a mRNA template, therefore, maintaining the relatively long-term effects of D-RNAi.

#### Mechanical Differences of PTGS/RNAi/D-RNAi in Initiation, Spreading, and Maintenance

We observed that the D-RNAi shares some similarities with PTGS/RNAi mechanisms (Fig. 3). At present, we do not know the mechanism(s) by which the transfection of a mRNA-cDNA hybrid rather than aRNA-

cDNA hybrid induces the long-term effect of gene silencing as reported here. We have detected a potential RdRp-dependent mechanism of D-RNAi, which may possess the ability to initiate and maintain, but not to spread, the effects of PTGS/RNAi. As shown in Fig. 4, it required at least two to three transfections to produce a complete apoptosis in LNCaP cells because the liposomal transfection method we used showed only a 30–40% transfection rate (24). These observations also indicate that there is no or less spreading effect caused by the D-RNAi. Grant *et al.* (1) have suggested that

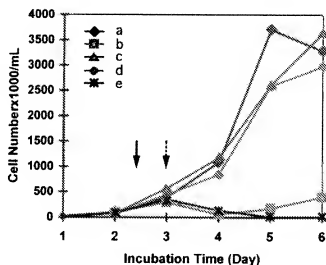


FIG. 4. Linear plot of interaction between incubation time (X) and cell growth number (Y). The black linear arrow shows the first addition of all tested probes, while the dotted arrow indicates the second addition of a mRNA-cDNA probe for double transfection analysis of D-RNAi. The proliferation rate of blank control (a), aRNA-cDNA (b) and ds-RNA (c) transfected cells was not affected, whereas the growth of mRNA-cDNA (d) transfected cells was remarkably inhibited after 36-h incubation ( $n = 4$ ). Because one transfection is not sufficient to reach the entire cell population, a more complete inhibition of cell growth was achieved after double transfections (e), indicating no spreading effect of D-RNAi.

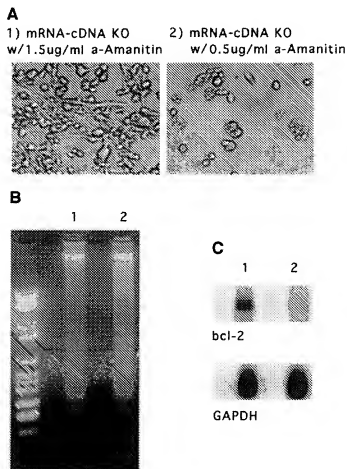
there are three major mechanical effects for PTGS, namely, initiation, spreading, and maintenance, which are also found in numerous inheritable RNAi phenomena. The initiation of D-RNAi may involve the onset of PTGS/RNAi which takes a relatively long period of time (1–3 days) to generate enough small RNAs or short aRNAs for specific gene knockout. For other traditional antisense transfection processes, it only takes several hours to reach the same gene silencing results but much higher dosages are required and frequently result in higher cytotoxicity.

Furthermore, unlike the effectiveness of the short-term traditional antisense transfections, the effects of PTGS/RNAi may spread from a transfected cell to its neighboring cells and can be maintained for a long period of time (weeks to lifetime) in the transfected cell and cells divided from the transfected cell. Indeed, the self amplification and regulation of PTGS/RNAi/D-RNAi make these specific gene interference phenomena unique to all previous gene silencing mechanisms. Conceivably, these systems seem to be involved in an intracellular defense system for eliminating unwelcome transgenes/foreign RNAs, particularly viral infections and retrotransposon activities (25). This type of defense system in human, however, has been overlooked, probably due to the fact that our current knowledge of the immune system is far more advanced and well-developed.

#### Identification of a Potential RdRp-like Enzyme for D-RNAi in LNCaP Cells

Since the RNA template-dependent mechanism of D-RNAi is most likely similar to the replication of some RNA viruses such as hepatitis D virus (HDV), it is speculated that one of the cellular RNA polymerases plays the role of RdRp in amplifying small RNAs or aRNAs for silencing genes. RNA polymerase II has been found to possess RNA-directed RNA synthesis activity (26, 27). More interestingly, as shown in Fig. 5, the addition of a low-dose of  $\alpha$ -amanitin (1.5  $\mu$ g/ml), an RNA polymerase II-specific inhibitor derived from a mushroom *Amanita phalloides* toxin, abrogated the apoptosis induced by bcl-2 D-RNAi. It is highly likely that the RNA polymerase II or an  $\alpha$ -amanitin-sensitive RNA-directed polymerase is responsible for the RdRp activity of D-RNAi in human prostate cancer LNCaP cells.

We have also observed that  $\alpha$ -amanitin concentration up to 3.5  $\mu$ g/ml caused partial transcriptional inhibition without significant apoptosis induction in the dehydrotestosterone-treated LNCaP cells. A remarkable inhibition of D-RNAi on bcl-2 has been detected although the  $\alpha$ -amanitin concentration we tested suppressed only 50% of the transcription activity. These findings suggest that the potential RdRp enzyme for D-RNAi in LNCaP cells is highly  $\alpha$ -amanitin-sensitive.



**FIG. 5.** Analysis of a potential D-RNAi-related RdRp enzyme by different  $\alpha$ -amanitin sensitivity: (1) 1.5  $\mu$ g/ml and (2) 0.5  $\mu$ g/ml. (A) The changes of cell proliferation rate and morphology after addition of  $\alpha$ -amanitin. A significant reduction of D-RNAi-induced apoptosis was detected in the 1.5 but not 0.5  $\mu$ g/ml  $\alpha$ -amanitin addition after mRNA-cDNA transfection ( $n = 3$ ), showing a dose-dependent release of cell growth inhibition. (B) Genomic ladder patterns demonstrated that the apoptotic induction effect of the bcl-2 mRNA-cDNA transfection was blocked by the 1.5  $\mu$ g/ml  $\alpha$ -amanitin addition. (C) Northern blots also showed that the bcl-2 silencing effect of D-RNAi had been prevented as well.

Since the replication of HDV is also a RNA-directed RNA synthesis procedure (26, 27), the binding of RNA polymerase II to a RNA template requires an A-T rich domain but not necessarily a TATA-box as in the transcription of a DNA template. The design of our bcl-2 RNA-DNA hybrids fits well with this requirement. The fragment of these probes is flanked behind the translation start codon of bcl-2 from +527 to +1293 nucleotide bases with multiple A-T(U) rich regions in the 3'-end of its sense orientation. However, the binding site of a RdRp-like enzyme to these probes and the exact function of the A-T rich domains remains to be determined.

Our finding of this D-RNAi phenomenon may provide a valuable new tool for PTGS/RNAi research in mammalian cells. Although these findings demon-

strate a novel mechanism quite different from PTGS/RNAi in other animal systems, a comparison between these different gene silencing effects will certainly shed more light on this novel intracellular system. The mechanisms by which PTGS/RNAi/D-RNAi phenomena take place and/or interactions among these various phenomena suggest a number of potential applications for future gene analysis and therapy. However, there are several issues still needing to be addressed, including the characteristics of RdRp enzyme, the interaction between RdRp and its templates, the RdRp product(s) responsible for the gene silencing effect, and RNases involved in the gene silencing effects. In addition, the relationship between the RdRp-derived product and the RNase, the differences among short-term, long-term and inheritable PTGS/RNAi/D-RNAi effects, and the limitation of these mechanisms regarding cell type, genomic status and gene activity certainly will be topics of further investigations.

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